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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 9/12, C07K 14/435, A61K 38/16, G01N 33/68	A1	(11) International Publication Number: WO 97/40142 (43) International Publication Date: 30 October 1997 (30.10.97)
(21) International Application Number: PCT/GB97/01152 (22) International Filing Date: 25 April 1997 (25.04.97) (30) Priority Data: 9608510.5 25 April 1996 (25.04.96) GB (71) Applicant (for all designated States except US): MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): NERI, Dario [IT/CH]; Institute Fuer Molekular Biologie und Biophysik, Eth-Hoenggerberg, CH-8093 Zürich (CH). WINTER, Gregory, Paul [GB/GB]; D4, Nevills Court, Trinity College, Cambridge CB2 1TQ (GB). (74) Agent: HOWARD, Paul, Nicholas; CarpmacIs & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: MODIFIED LIGANDS OF CALCIUM-DEPENDENT BINDING PROTEINS		
(57) Abstract		
<p>The present invention relates to ligands capable of binding a calcium dependent binding protein, that comprise an amino acid sequence corresponding to that of a wild type ligand for the calcium dependent binding protein, with a modification which results in enhanced affinity of the ligand for the calcium dependent binding protein.</p>		

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MODIFIED LIGANDS OF CALCIUM-DEPENDENT BINDING PROTEINS

Introduction

The present invention relates to targeting, detection,
5 immobilization, and purification of molecules using binding
pairs. In particular, it relates to the use of calcium
dependent binding proteins and ligands thereof. The present
invention provides ligands that possess improved affinity
for calcium-dependent binding proteins. The binding pairs of
10 the present invention provide an attractive alternative to
currently available binding pair systems utilised in
biological procedures.

Background to the invention

15 The term "binding pair" refers to two molecules that bind
each other with high affinity. Many binding pair systems
presently exist for the purification and targeting of tagged
compounds. The most widely-used system at present is the
biotin-streptavidin binding pair that has also been applied
20 to *in vivo* tumour targeting methods (Paganelli et al.,
1991). In addition, recombinant fusion proteins expressing
the myc or Flag tags have been isolated and purified using
antibodies directed against these tags (Munro and Pelham,
1986; Hopp et al., 1988). Poly-his peptide tails have been
25 genetically fused to recombinant proteins and purified on
nickel-coated agarose or using affinity columns (Skerra et
al., 1991). The streptavidin-binding strep tag (Schmidt and
Skerra, 1993) has been used in similar applications.

30 However, the application of each of the above examples is
restricted. Techniques such as affinity purification require
a specific yet low affinity interaction so as not to impair
the function of the recombinant protein with a harsh elution
protocol. Conversely, targeting and specific immobilization
35 both require high affinity binding of the tag with a long
half-life of interaction of the binding pair. The systems
described in the prior art that employ binding pairs do not
possess this flexibility, and thus are not universally

applicable.

Calmodulin is a calcium dependent binding protein that regulates a wide range of enzymes, and plays a key role in
5 intracellular signal transduction. It is also one of the few examples of a small protein capable of binding ligands with a high affinity.

Due to their small size, calmodulin-ligand complexes have
10 been used as convenient models for the study of protein association and dissociation. Since the elucidation of three-dimensional structures of calmodulin in complex with high affinity ligand substrates, this system has been amenable to the study of the mechanisms by which proteins
15 recognise one another in high affinity interactions.

Pending patent application W095/12672 discloses the use of binding pairs comprising calmodulin and calmodulin ligands for the detection, immobilization, targeting and
20 purification of recombinant polypeptides. This system is attractive since the binding interaction can be modified through changes in calcium concentration that can easily be controlled through the use of calcium chelators. This greatly broadens the applicability of binding pairs as
25 experimental and therapeutic tools.

Stofko-Hahn et al., (1992) have made a fusion of a calmodulin binding ligand tag derived from the C terminus of rabbit skeletal muscle myosin light chain kinase (sMLCK),
30 and a recombinant protein. They have used this in purification strategies on an affinity support. In the presence of high levels of calcium, this system displays a specific high affinity interaction, the dissociation constant for the binding pair being of the order of 3nM. The
35 addition of EGTA (a calcium chelator) lowers the affinity of the interaction, allowing a very mild elution protocol, and meaning that denaturation or disruption of the native protein structure is unlikely. However, although the

affinity of calmodulin towards calmodulin ligands is high, it is not sufficiently high for many targeting, immobilisation and particularly therapeutic applications.

- 5 For many therapeutic applications, sub nanomolar or picomolar dissociation constants, high association rate constants and extremely low dissociation rate constants are necessary to ensure efficient targeting of the complex and a sufficiently long half-life of the binding interaction.
- 10 The biotin-streptavidin binding pair is widely used both experimentally and therapeutically for the targeting, purification and immobilization of proteins. The main attraction of this system lies in the high affinity of interaction ($K_d = 10^{-15}M$) of the binding pair.

15

- However, a significant disadvantage of using this binding pair is the immunogenicity of streptavidin in the human body. This greatly limits the utility of this system for therapeutic applications. There is thus a great need for an
- 20 experimentally malleable binding pair system that possesses both a high affinity interaction and low immunogenicity.

Description of the invention

- According to the present invention, there is provided a
- 25 ligand capable of binding a calcium dependent binding protein comprising an amino acid sequence corresponding to that of a wild type ligand for the calcium dependent binding protein, with a modification which results in enhanced affinity of the ligand for the calcium dependent binding
- 30 protein.

- By wild type ligand is meant a naturally-occurring ligand capable of binding a calcium dependent binding protein. All ligands as defined by the present invention are peptides
- 35 that comprise a modification of the binding domain of naturally-occurring peptide ligands that interact with calcium dependent binding proteins.

Examples of wild type ligands from which suitable ligands may be derived are skeletal myosin light chain kinase, smooth muscle myosin light chain kinase, mastoparan, melittin, AC-28 and NO-30, all of which exhibit high
5 affinity binding to calmodulin. Preferably, the ligand of the present invention comprises a modified skeletal myosin light chain kinase ligand.

The said modification may consist of a substitution,
10 insertion, or deletion of one or more amino acids in the wild type ligand that results in improved affinity of the ligand for the calcium dependent binding protein. The ligand of the present invention may comprise up to 20, preferably up to 10, more preferably up to 5 modifications to the amino
15 acid sequence of the wild type ligand.

By insertion and deletion is meant the introduction or omission respectively of one or more amino acids, to lengthen or shorten the amino acid sequence of the wild type
20 ligand, and thus modify its affinity for the calcium dependent binding protein. Preferably the modification comprises a substitution of one or more amino acids in the wild type ligand.

25 A substitution comprises the replacement of a naturally-occurring amino acid in the wild type peptide sequence for a surrogate residue. The preferred surrogate residue is alanine or valine. Both these amino acids are hydrophobic with relatively innocuous side chains, and both are thus
30 ideal for substitution into a helical peptide with minimal perturbation of the peptide structure. Fully worked examples describing the production, identification and manipulation of such molecules are disclosed herein. Preferably the surrogate residue is alanine.

35

Individual alanine residues have previously been inserted in the sequence of proteins. Clackson and Wells, (1995) recently reported the systematic replacement of contact

residues in the sequence of the human growth hormone receptor (hGHbp). This "alanine scanning" technique identified the residues involved in the binding interaction through quantification of the decrease in binding affinities
5 exhibited by these mutant proteins.

Barstar, a physiological inhibitor of barnase (the extracellular RNase in *Bacillus amylolique-faciens*), has also been subjected to such experimental manipulation. The
10 very high association rate constant possessed by the barnase-barstar complex ($3.8 \times 10^9 \text{ s}^{-1} \text{ M}^{-1}$) is due to four acidic sidechain amino acid residues. When these were all individually mutated to alanine, this caused a decrease in the association rate constant. Concomitantly, a slight
15 increase in the stability of barstar was seen, and it has therefore been hypothesized that proteins evolve primarily to optimize their function rather than their stability (Schrieber et al., 1994).

20 Surprisingly, the modified ligands of the present invention exhibit increased binding affinities and association constants. It seems that ligands capable of binding calcium dependent binding proteins have evolved to perform their biological function by achieving sufficiently high binding
25 affinity, yet have failed to maximise binding affinity.

Multiple amino acid substitutions are also claimed in the present invention, and described herein. The combination of individual single amino acid substitutions can give a
30 cumulative effect, leading to a much enhanced affinity of the ligand for the calcium dependent binding protein.

By calcium dependent binding protein it is meant a protein capable of binding a ligand wherein the dissociation
35 constant for the ligand is reduced in the presence of calcium ions, that is, binding is much stronger in the presence of calcium ions.

The ligand of the present invention is suitable for many biotechnological applications, for example for protein purification and detection, conditional formation of bifunctional (macro)molecules, microscopy, FACS analysis, protein immobilisation on microsensor chips and tumour targeting. For many of these applications it is desirable for the calcium dependent binding protein-peptide ligand binding pair to have a very high affinity, with a fast on-rate of association, and a very slow off-rate (ie. dissociation rate constant).

For the applications described herein, it is preferred that calcium dependent reduction in binding affinity is at least ten-fold where the binding protein has one tenth of its calcium ion sites occupied. Preferably, the dissociation constant is greater than 10nM at a pH of between 6 and 9 at 20°C, and 10nM or less in the presence of 50µM calcium ions, most preferably 1nM or less. For some calcium dependent binding proteins, other analogous ions may replace calcium, for example strontium.

The preferred calcium dependent binding protein is calmodulin. This protein is relatively small (148 residues) and is thus suitable for use as one member of a binding pair. Hybrid recombinant proteins of calmodulin and another protein may be easily generated by genetic fusions; the use of a small protein as a tag reduces the risk of perturbing the protein structure of the fusion partner, and also reduces the chance of abrogating the function of that partner through steric hindrance.

It will be understood that other calcium-dependent binding proteins such as troponin C, calcineurin, parvalbumin and oncomodulin may also be employed in the present invention.

Several proteins, peptides, or organic compounds bind to calmodulin with high affinity (with a nM or sub nM dissociation constant). Indeed, three-dimensional structures

are available of calmodulin and peptide ligands (Ikura et al., 1992; Meador et al., 1992, 1993). The NMR structure of the complex between calmodulin and a 26 amino acid peptide derived from skeletal myosin light chain kinase (sMLCK) has
5 shown that only the central 19-mer sequence RWKKNFIAVSAANRFKKIS contacts calmodulin (Ikura et al., 1992). In the present invention, the preferred wild type peptide ligand comprises a modification of this sequence, as described further herein.

10

It is preferred that the ligand of the present invention shows a reduction in K_d by a factor of at least 10, preferably by a factor of at least 100, more preferably by a factor of at least 1000, over the wild type ligand,
15 wherein

$$K_d = k_{off}/k_{on}$$

20 where k_{off} = the kinetic dissociation constant of the reaction

and k_{on} = the kinetic association constant of the reaction

The decrease in dissociation constant may be derived from
25 modification of either the k_{off} or k_{on} values, or through a combination of modifications in both kinetic values. Preferably, the improvement in binding affinity reflected by a lowering of the dissociation constant is dominated by a lowered k_{off} value, by a factor of at least 10, more
30 preferably at least 100 and most preferably by a factor of at least 1000. The binding affinity is also reflected in the half-life (t_h) of interaction, wherein

$$t_h = 1/k_{off}.$$

35

Preferably the half-life of the interaction is at least 15 minutes, more preferably at least one hour.

Peptides 6, 10, and 17 in the accompanying Table 1 show the

greatest improvement in K_d over the wild type peptide. In particular, peptide 6 is the ligand of choice.

According to a further aspect of the present invention there is provided a method of enhancing the binding affinity of a wild type calcium dependent binding ligand comprising the step of modifying the amino acid sequence of the wild type calcium dependent binding ligand.

10 The ligands of the present invention may be synthesized by conventional peptide synthetic techniques including solid phase oligopeptide synthesis and recombinant DNA techniques.

Robotic multi-well peptide synthesizers allow the production of many different peptides comprising multiple amino acid substitutions in 1-2 days. Parallel methods of peptide deprotection, cleavage and fluorescent labelling with multi-well devices, allow rapid analysis of the peptides generated. The native gel electrophoresis method used herein for the measurement of kinetic dissociation constants by competition experiments can also be used in parallel with peptide production, using multiple multi-well gels.

In addition, libraries of peptides can be designed and expressed on the surface of filamentous phage by recombinant DNA techniques (Smith et al., 1985). Suitable stringent selection/amplification and screening techniques (for example parallel fluorescent native gel electrophoresis) can be used to identify improved ligands.

30

According to a further aspect of the present invention, there is provided a binding pair comprising a calcium dependent binding protein and a ligand according to the present invention.

35

According to the present invention there is provided use of a ligand according to the present invention in conjunction with a calcium dependent binding protein as a binding pair.

The calcium dependent binding protein-ligand binding pair of the present invention may be employed in any application requiring a high affinity binding pair. Modification of the binding affinity according to the present invention allows
5 the binding affinity of the binding pair to be tailored to the requirements of the specific application.

According to a further aspect of the present invention, the ligand of the present invention may be linked with another
10 moiety. For example, co-pending United Kingdom patent application "Isolation of enzymes" filed in the name of the Medical Research Council on 25th April 1996 discloses the use of calmodulin linked to an enzyme. In addition, calmodulin ligands are linked to substrate for the
15 particular reaction catalysed by the enzyme. Following binding of the ~~calmodulin-enzyme~~ moiety and the ligand-substrate moiety and conversion of substrate to product by the enzyme, the product remains bound to the enzyme (via the calmodulin-calmodulin ligand binding pair) after the product
20 has dissociated from the active site. This allows the isolation of active enzyme from an enzyme library, by selection of the product of the enzymatic reaction. If the enzyme is linked to the DNA encoding it (for example by being displayed on the surface of phage), isolation of the
25 encoding DNA species is also possible.

The present invention may be used to increase the binding affinity and thus half-life of the interaction of the calmodulin-calmodulin ligand binding pair used in the above-
30 mentioned procedure. This is advantageous particularly in the case of enzymes that possess a slow rate of reaction. For these enzymes, the calmodulin-ligand interaction must exhibit a sufficiently long half-life to allow the completion of the enzymatic reaction, so as not to allow
35 dissociation of substrate from the enzyme. The use of the ligands of the present invention will broaden the application of this "enzyme selection" technology.

The linked moiety may be a protein. Preferably the ligand-protein fusion is produced from a gene fusion. Nucleic acid encoding such fusions forms a further aspect of the present invention. Host cells transfected with an expression vector
5 encoding such a recombinant fusion protein may be grown in culture and used for the production of such molecules. The ligand may then be used for the isolation and purification of the recombinant molecules, by processes such as affinity chromatography or ion exchange chromatography on
10 (calmodulin) antagonist columns. Elution may make use of a calcium chelator to lower the affinity of calmodulin for the ligand.

Alternatively, the linked moiety may be appended to the
15 ligand non-covalently, or by chemical modification. Chemical modification includes, for example, linking reactive functional groups such as thiol, hydroxy, amino, carboxy, or aryl groups present in the ligand to reactive functional groups present in the linked moiety. For instance, a
20 cysteine group can be incorporated at the C-terminus of the ligand, and a protein coupled using a heterobifunctional crosslinker such as SPDP (N-succinimidyl 3 (2-pyridyldithio)propionate). For non-covalent linkage, peptides favouring formation of dimers (such as leucine
25 zippers or the protein binding domains of Jun and Fos) may be appended to the peptide, and to the recombinant protein. The complex thus forms on mixing of the protein and ligand.

The linked moiety may also be a label. The label may be
30 fluorescent (eg. fluorescein), an antibody label, or a radiolabel. This permits detection and isolation of recombinant fusion proteins comprising a calcium dependent binding protein by methods such as fluorescence activated cell sorting (FACS), confocal fluorescence microscopy or
35 blotting. Other highly sensitive methods for detection of calcium dependent binding proteins may be developed, for example by Tb³⁺ luminescence after replacing Ca²⁺ ions with Tb³⁺ ions as recently described for oncomodulin. (Clark et

al., 1993). Through the use of a labelled ligand of the present invention, measurements of binding affinity may also be made using a band shift assay as described in co-pending patent application WO95/12672.

5

A variety of techniques are under development to improve the efficacy and tumour cell specificity of cancer treatment. Chemotherapy has proven to display a low therapeutic index, which causes unacceptable damage to normal organs, and
10 limits the dose of drug that can safely be administered.

For many years, antibodies have been investigated as means to deliver cytotoxic proteins to kill target cells, and indeed, the majority of research is at present directed
15 towards antibody-directed therapy. The cell specificity of antibodies or their immunoreactive fragments can be harnessed to deliver drugs, radioisotopes, protein cytotoxins, effector cells of the immune system, and even enzymes for targeted prodrug activation. However, despite
20 outstanding results *in vitro* and *in vivo*, considerable problems remain, such as the immunogenicity of the molecule(s) and systemic toxicity, that must be resolved before effective clinical trial results may be obtained.

25 One method that attempts to resolve the systemic toxicity associated with direct antibody-mediated delivery is the pre-targeted delivery of therapeutic agents using streptavidin and biotin based conjugates. The target-specific agent (normally an antibody) is fused to
30 streptavidin and administered to a mammal. After a relevant localisation time, a second conjugate is administered that comprises biotin linked to a toxin or radioisotope. Thus, the targeting ratio and/or active dose of the cytotoxic agent is increased at the target organ. In addition,
35 exposure to non-target tissues (especially bone marrow) is reduced, and the delivery and efficient utilisation of larger doses becomes possible.

Non-targeted label is efficiently excreted from the body through the kidneys. However, the degree of efficacy of this therapy is markedly reduced by the immunogenicity of streptavidin, and thus also of its conjugates. This
5 necessitates the simultaneous administration of an immunosuppressant, an inefficient measure that raises the degree of trauma associated with the therapy. There is thus a need for an analogous system comprising a binding pair that exhibits both high binding affinity and low
10 immunogenicity in the mammalian body.

Calcium dependent binding proteins such as calmodulin and peptide ligands therefor are thus ideal candidates for such a therapeutic regime due to their natural occurrence in the
15 mammalian body, and thus low immunogenicity (VanEldik and Lucas, 1987). In addition, it has been shown that calmodulin is not toxic, does not accumulate selectively in any organ, and is secreted rapidly in the urine. However, the interaction of calmodulin with naturally-occurring peptide
20 ligands is of too low affinity for effective use in this application.

The present invention provides a peptide ligand of improved affinity for calmodulin. Thus, another embodiment is a
25 molecule comprising the ligand of the present invention linked to a toxin. This toxin may comprise drugs, a radioisotope, a protein cytotoxin, an effector cell of the immune system, an enzyme for prodrug activation, a *Pseudomonas* toxin, tumour necrosis factor alpha, or another
30 toxin with a chemotherapeutic action. It will be understood that any suitable toxin may be used as a fusion partner in the present invention for pretargeted delivery of therapeutic agents.

35 The same technique, only involving the fusion of the ligand to a radiolabel, may be used in immunoscintigraphy for the diagnosis and imaging of tumours.

In addition to use of the ligand as the labelled or conjugated molecule in the embodiments described above, the format may be reversed so that the moiety fused to the toxin or label is the calcium dependent binding protein molecule, and the entity to which this is targeted is the peptide of the present invention. All the concepts described above are thus innately reversible.

In addition, fragments of these ligands and calcium dependent binding protein molecules that retain binding activity may be used, as may their derivatives. The term "derivative" encompasses amino acid variants containing deletions, substitutions, or insertions, that lead to altered physical or chemical molecular properties.

The strong binding interactions between calcium dependent binding proteins and peptide ligands may also provide a means of creating dimers or multimers. Dimeric peptide ligands could be used to dimerise recombinant calcium dependent binding protein fusion molecules. In the same way, multimeric peptide ligands would be capable of producing multimers. This would be particularly applicable in the case of calmodulin-antibody multimers formed using such ligands that could be used to increase the efficiency of tumour targeting, and thus the efficacy of the immune response.

Various aspects and embodiments of the present invention are illustrated in the following examples with reference to the figures. Further aspects and embodiments of the present invention will be apparent to those skilled in the art.

All documents mentioned in the text are incorporated by reference.

Figure 1 illustrates detection of the complex between calmodulin and fluorescein-labelled peptides by native polyacrylamide gel electrophoresis.

The number of the lane corresponds to the peptide number. The position of the substitution is indicated with a line connecting the gel lane and the amino acid sequence.

- 5 **Figure 2** illustrates typical sensograms of calmodulin binding to peptides 1-17.

Here the results are shown for peptides 1 and 5.

- 10 **Figure 3** illustrates competition experiments for the measurement of k_{off} constants for peptides 1-17 towards calmodulin.

Here the results of the competitions are shown for peptides 15 1, 2, 3, 6, 9 and 14. Incubation times (in minutes) are indicated under the lanes.

The following examples are provided by way of example only. It will be appreciated that modification of detail may be 20 made without departing from the scope of the invention.

Examples:

Example 1: Design and synthesis of peptides.

- 25 It has previously been shown that in skeletal myosin light chain kinase, only the central 19-mer sequence RWKKNFIAVSAANRFKKIS contacts calmodulin. A modification of this peptide was therefore chosen as a model for the design of peptides directed against the calmodulin binding site.

30

Peptide 1 contains the wild type sequence and was designed using an N terminal cysteine to act as a target for site-specific peptide functionalisation with thiol-specific reagents (such as iodoacetamido fluorescein or biotin 35 derivatives). The following three alanines are spacers, and at the C terminal, an amide group is provided. The sequence of this peptide is thus CAAA-RWKKNFIAVSAANRFKKIS-CONH₂.

16 further peptides were synthesized corresponding to
alanine mutations of all the non-alanine residues in the
calmodulin recognition sequence. The sequences of these
peptides are shown in Table 1, which also provides data on
5 the kinetic analysis of the interaction between calmodulin
and calmodulin-binding peptides by individual amino acid
replacement to alanine.

Peptides were made on a solid phase using a multiple peptide
10 synthesizer (Multisyn Tech, Bochum, Germany) employing
Fmoc/t-butyl protecting groups. The Fmoc group was cleaved
by 40% (v/v) piperidine in dimethylformamide and successive
amino acids were added as N-hydroxybenzotriazole esters. The
peptides were deprotected and cleaved from the resin using
15 93% trifluoroacetic acid; 3% ethanedithiol; 2% anisole; 2%
water. Peptides were analyzed by HPLC using a Vydac C18
column (10 μ M, 100 x 250mm) and by amino acid analysis (PICO
TAG, Waters, Milford, MA).

20 Example 2: Detection of the complex between calmodulin and
fluorescein-labelled peptides by native polyacrylamide gel
electrophoresis (PAGE).

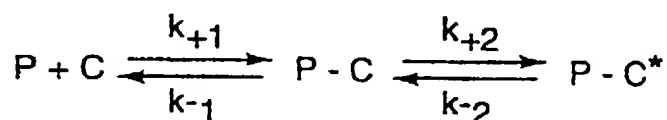
Complexes between calmodulin from bovine brain (1 μ M, Sigma)
25 and fluorescein-labelled sMLCK-derived peptides (as
described in Table 1) were prepared in gel buffer (25% gel
mix [4g sucrose + 1mg bromophenol blue in water to give a
10ml solution] + 75% TBSC [50mM TRIS pH 7.4, 100mM NaCl +
50 μ M CaCl₂]) and run on a 15% native PAGE gel (5ml 30%
30 acrylamide-bisacrylamide solution + 4.5ml water + 0.5ml 3M
Tris, pH8.8, + 1 μ l 1M CaCl₂ polymerised with 30 μ l 25%
ammonium persulphate and 9 μ l TEMED) using 14.4g/l glycine +
3g/l Tris-base + 0.1mM CaCl₂ as running buffer. Peptides
were labelled either with iodoacetamido fluorescein
35 (Molecular Probes) or with iodoacetamido-LC-biotin (Pierce)
essentially as previously described by Neri et al., (1995).
In these conditions, the fluorescence of the positively-
charged labelled peptides can be detected only if they form

a stable complex with calmodulin.

Figure 1 shows that all the synthesized peptides bind to calmodulin in native PAGE gels, indicating that single alanine substitutions have no severely deleterious effect on calmodulin binding. The number of the lane corresponds to the peptide number. The position of the substitution is indicated with a line connecting the gel lane and the amino acid sequence. No fluorescent band can be detected with non-correlated labelled peptides (data not shown). The gels were imaged with the chemiluminescence analyzer LUANA (Neri et al., 1996).

Example 3: Measurement of the isomerization, association and dissociation kinetics of calmodulin-binding peptides.

It has been shown by Török and Trentham (1994) that smooth muscle MLCK-derived peptides bind to calmodulin with a biphasic kinetic according to the scheme:



in which P is the peptide, C is calmodulin, P-C is the first complex formed on association of P and C, which then undergoes a conformational isomerisation to P-C*.

Stopped-flow analysis of calmodulin binding to the sMLCK-derived peptides of the present invention shows a biphasic binding kinetic (data not shown). For a kinetic and thermodynamic characterisation of the binding of the peptides of the present invention to bovine brain calmodulin, we have measured the kinetic isomerisation, association, and dissociation constants from which dissociation constants can be derived. This allows assessment of those mutations that have resulted in

increased affinity towards calmodulin.

$$k_{\text{isom}} = k_{+2} + k_{-2} \quad [1]$$

$$k_{\text{on}} = k_{+1} \quad [2]$$

$$5 \quad k_{\text{off}} = k_{-1} \cdot k_{-2} / (k_{+2} + k_{-2}) \quad [3]$$

$$K_d = k_{\text{off}} / k_{\text{on}} \quad [4]$$

k_{isom} constants have been measured by stopped-flow, detecting changes in tryptophan fluorescence for all the peptides except 3, in which the tryptophan is replaced by alanine. These values are shown in Table 1.

Real-time interaction analysis by surface plasmon resonance on a BIAcore instrument (Jönsson et al., 1991) was used to determine k_{on} and k_{off} constants. Biotinylated peptides 1-17 were bound to commercially available streptavidin-coated microsensor chips (Pharmacia Biosensor), in order to achieve a surface capacity of 100-200 surface plasmon resonance units of calmodulin bound. Different concentrations of calmodulin (5, 10, 20, 50 and 100nM in the order of Figure 2) were injected and allowed to associate with the peptides. Regeneration of the surface was achieved by injection of 5 μ l TBS + 15mM EDTA which dissociates the calmodulin-peptide complex. Figure 2 shows sensograms of peptides 1 and 5, in which the difference in k_{on} and k_{off} between the two peptides can be visually appreciated. k_{on} and k_{off} constants were obtained from the sensograms using the BIAevaluation software version 2.1 according to the manufacturers instructions (Pharmacia Biosensor). The results of the BIAcore measurements are listed in Table 1.

Kinetic association constants (k_{on}) of sMLCK-derived peptides 1-17 are in the range $6.5 \times 10^5 - 3.2 \times 10^6 \text{ s}^{-1} \text{M}^{-1}$, and are more than 100-fold lower than those of smooth muscle MLCK-derived peptides (Török and Trentham, 1994). The largest increase in k_{on} with respect to the wild-type peptide 1 was observed for R5 (peptide 2), W6 (peptide 3) and K20 (peptide 14) mutations to alanine (4-5 fold increase; Table 1). Thus

TABLE 1

KINETIC ANALYSIS OF THE INTERACTION BETWEEN CALMODULIN AND CALMODULIN-BINDING PEPTIDES
BY INDIVIDUAL AMINOACID REPLACEMENT TO ALANINE

Peptide Sequence:			(boldface: single-point mutations to alanine in individual peptides)				
0	1	2					
1	-----0-----	0----					
CAAARWKKNFIAVSAANR FKK IS							
pept.	mutation	k _{isom.} (s ⁻¹)A	k _{on} (s ⁻¹ M ⁻¹)B	k _{off} (s ⁻¹)C	k _{off} (s ⁻¹)B	K _d (M)D	
1	w1	1.9 (±0.7) x 10 ⁻¹	6.5 (±1.6) x 10 ⁵	≥ 1 x 10 ⁻³	2.4 (±0.6) x 10 ⁻³	3.7 (±1.8) x 10 ⁻⁹	
2	R5-->A	1.2 (±0.5) x 10 ⁻¹	3.0 (±0.7) x 10 ⁶	≥ 1 x 10 ⁻³	1.5 (±0.4) x 10 ⁻³	5.0 (±2.5) x 10 ⁻¹⁰	
3	W6-->A	N.D.	3.2 (±0.8) x 10 ⁶	≥ 1 x 10 ⁻³	5.0 (±1.2) x 10 ⁻³	1.6 (±0.8) x 10 ⁻⁹	
4	K7-->A	2.4 (±0.6) x 10 ⁻¹	1.3 (±0.3) x 10 ⁶	≥ 1 x 10 ⁻³	3.0 (±0.7) x 10 ⁻³	2.3 (±1.1) x 10 ⁻⁹	
5	K8-->A	7.4 (±1.0) x 10 ⁻²	1.1 (±0.3) x 10 ⁶	2.1 (±1.0) x 10 ⁻⁴	2.9 (±0.7) x 10 ⁻⁴	2.6 (±1.3) x 10 ⁻¹⁰	
6	N9-->A	1.8 (±0.9) x 10 ⁻¹	9.8 (±2.5) x 10 ⁵	2.2 (±1.1) x 10 ⁻⁶	< 1 x 10 ⁻⁴	2.2 (±1.4) x 10 ⁻¹²	
7	F10-->A	2.5 (±0.7) x 10 ⁻¹	9.0 (±2.2) x 10 ⁵	6.0 (±3.0) x 10 ⁻⁴	3.9 (±1.0) x 10 ⁻⁴	4.3 (±2.1) x 10 ⁻¹⁰	
8	I11-->A	1.1 (±0.3) x 10 ⁻¹	9.2 (±2.3) x 10 ⁵	4.7 (±2.3) x 10 ⁻⁴	2.3 (±0.6) x 10 ⁻⁴	2.5 (±1.2) x 10 ⁻¹⁰	
9	V13-->A	1.5 (±0.3) x 10 ⁻¹	8.6 (±2.1) x 10 ⁵	≥ 1 x 10 ⁻³	1.2 (±0.3) x 10 ⁻³	1.4 (±0.7) x 10 ⁻⁹	

10	S14-->A	2.1 (± 1.0) $\times 10^{-1}$	1.1 (± 0.3) $\times 10^6$	7.9 (± 3.9) $\times 10^{-5}$	1.0 (± 0.2) $\times 10^{-4}$	9.1 (± 4.5) $\times 10^{-11}$
11	N17-->A	2.8 (± 0.6) $\times 10^{-2}$	9.4 (± 2.3) $\times 10^5$	4.9 (± 2.4) $\times 10^{-4}$	3.5 (± 0.9) $\times 10^{-4}$	3.7 (± 1.8) $\times 10^{-10}$
12	R18-->A	1.7 (± 0.4) $\times 10^{-1}$	1.6 (± 0.4) $\times 10^6$	8.3 (± 4.1) $\times 10^{-4}$	4.1 (± 1.0) $\times 10^{-4}$	1.8 (± 0.9) $\times 10^{-10}$
13	F19-->A	1.8 (± 0.8) $\times 10^{-1}$	9.3 (± 2.3) $\times 10^5$	$\geq 1 \times 10^{-3}$	8.4 (± 2.1) $\times 10^{-4}$	9.0 (± 4.5) $\times 10^{-10}$
14	K20-->A	1.6 (± 0.2) $\times 10^{-2}$	2.5 (± 0.6) $\times 10^6$	1.5 (± 0.7) $\times 10^{-4}$	1.4 (± 0.4) $\times 10^{-4}$	5.6 (± 2.8) $\times 10^{-11}$
15	K21-->A	1.2 (± 0.3) $\times 10^{-1}$	1.1 (± 0.3) $\times 10^6$	2.1 (± 1.0) $\times 10^{-4}$	2.2 (± 0.5) $\times 10^{-4}$	2.0 (± 1.0) $\times 10^{-10}$
16	I22-->A	8.7 (± 1.5) $\times 10^{-2}$	8.1 (± 2.0) $\times 10^5$	2.8 (± 1.4) $\times 10^{-4}$	3.1 (± 0.8) $\times 10^{-4}$	3.8 (± 1.9) $\times 10^{-10}$
17	S23-->A	1.3 (± 0.4) $\times 10^{-1}$	8.2 (± 2.0) $\times 10^5$	7.0 (± 3.5) $\times 10^{-5}$	1.3 (± 0.3) $\times 10^{-4}$	1.6 (± 0.8) $\times 10^{-10}$

Legend To Table 1:

A) k_{isom} (s^{-1}) measured by stopped-flow as described in Figure 2

B) k_{on} ($\text{s}^{-1}\text{M}^{-1}$) and k_{off} (s^{-1}) measured by BIAcore as described in Figure 2. Only a condition is reported for k_{off} (s^{-1})B

of peptide 6. This is due to the fact that measurement of very slow dissociation constants by BIAcore is made difficult by rebinding effects and by baseline instability, particularly when, like in the present study, calcium ions are used and the microsensor chip is derivatised to low surface density.

C) k_{off} (s^{-1}) values measured by competition as described in Figure 2

D) K_d (M) = k_{off} (s^{-1})B / k_{on} ($\text{s}^{-1}\text{M}^{-1}$)B, except for peptide 6 for which $K_d = k_{\text{off}}$ (s^{-1})C / k_{on} ($\text{s}^{-1}\text{M}^{-1}$)B

N.D. = Not Determined

alanine mutations of two positively-charged amino acid residues (R5 and K20) are associated with increased on-rates.

- 5 Certain alanine mutations improve k_{off} values up to 1 000-fold with respect to the wild-type peptide 1 (Table 1). The largest improvements are observed for the replaced hydrophillic residues (N9, S14, S23), which are not stable in the hydrophobic environment of the calmodulin core. These
10 correspond to peptides 6, 10, and 17.

Example 4: Determination of k_{off} constants by competition experiments.

- 15 In order to obtain an independent experimental confirmation of the BIAcore results, k_{off} constants for peptides 1-17 towards calmodulin were determined by competition experiments. 30nM fluorescein-labelled peptide/calmodulin complexes in gel buffer were competed at room temperature
20 for different times with 30-fold excess of unlabelled peptide. The resulting mixtures were run on native PAGE gels and imaged by LUANA as described above. The bands in the image obtained were integrated using the LUANA software (Neri et al., 1996) and the corresponding intensities
25 plotted versus time and fitted with a single exponential, from which k_{off} constants were derived. In order to normalise band intensities against pipetting errors, the samples contained 6nM free fluorescein which ran with the front. The fluorescein bands were integrated and used to normalise the
30 calmodulin/fluorescent peptide complex band intensity. The results of competitions for peptides 1, 2, 3, 6, 9, and 14 are shown in this figure 3. Incubation time in minutes is indicated under the lanes.
- 35 In the fast off-rate extreme, peptides 1, 2, 3 and 9 dissociate from calmodulin in a few minutes. Peptide 14 is representative of an intermediate off-rate, whereas 6 is hardly competed by an excess of unlabelled peptide after

1000 minutes. The volumes of the bands were plotted versus competition time and fitted to a single exponential, from which k_{off} values were derived. The values are shown in Table 1.

5

The fastest competitions (peptides 1-4, 9 and 13) were over by the time the calmodulin/fluorescent peptide complex entered the gel (approximately 5-10 minutes from pipetting a molar excess of unlabelled peptide; Figure 3), from which
10 a condition on the off-rate constant $k_{off} > 1 \times 10^{-3} \text{ s}^{-1}$ was derived. Even for these fast off-rates, complexes can be detected by native polyacrylamide gel electrophoresis because of the well known gel "cage effect" (Fried and Crothers, 1981; Garner and Revzin, 1981).

15

The k_{off} values measured by competition and by BIAcore are in substantial agreement (Table 1). Small differences between the two sets of data may be due to the fact that competition experiments were performed in gel mix buffer
20 (Figure 3) rather than in TBS + 50 μM CaCl_2 .

Example 5: Construction of further mutations.

Due to the enhanced binding affinity for calmodulin shown by
25 peptide 6 over all other tested peptides, further single point mutations of the asparagine residue 9 of the wild type peptide were constructed. The sequence of these peptides is shown in table 2, along with the K_D values, measured according to the methods described in Examples 3 and 4.
30 Hydrophobic donor residues (valine, phenylalanine and leucine) were chosen because it was hypothesized that these residues would contribute to the stability of the hydrophobic environment in the calmodulin core.

35 All of the designed peptides possessed greater affinity for calmodulin than did the wild type SMLCK peptide, although none of the three showed greater affinity than peptide 6.

TABLE II
 kinetic analysis of the interaction between calmodulin and calmodulin-binding peptides by replacement of N9 to various hydrophobic residues.

peptide	mutation	K_{on}	K_{off}	K_D (M)
wild type	wt	6.1×10^5	5.9×10^{-6}	$3.9 (\pm 1.8) \times 10^{-9}$
a	N9→V	8.5×10^5	3.5×10^{-4}	1×10^{-9}
b	N9→F	2.9×10^5	2.9×10^{-5}	1×10^{-10}
c	N9→L	1.1×10^6	2.3×10^{-5}	3×10^{-11}

Example 6: Construction of multiple mutations.

The mutant peptides that exhibited the greatest improvement in affinity for calmodulin were peptides 6, 10 and 14, corresponding to alanine substitutions at positions 9, 14 and 20 respectively. A multiple mutant was constructed in which these three residues were altered to alanine. The sequence was as shown below, with the replacement residues shown in bold:

10

CAAARW**K**KAFIAVAAAN**R**FAKIA

Using the methods of examples 3 and 4, the dissociation constant of this peptide for calmodulin was found to be 1×10^{-11} M ($k_{on}=6.1 \times 10^5$; $k_{off}=5.9 \times 10^{-6}$). The affinity of this peptide for calmodulin is therefore comparable to that of peptide 6. However, no synergistic effect was achieved by mutating all of the hydrophilic residues in the peptide.

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April 1996.

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WO95/12672 Neri, D., Winter, G. P. and de Lalla, C.

CLAIMS

1. A ligand capable of binding a calcium dependent binding protein comprising an amino acid sequence corresponding to
5 that of a wild type ligand for the calcium dependent binding protein, with a modification which results in enhanced affinity of the ligand for the calcium dependent binding protein.
- 10 2. A ligand according to claim 1 in which the calcium dependent binding protein is calmodulin.
3. A ligand according to claim 2 in which the said modification comprises the substitution, insertion or
15 deletion of one or more amino acids in the wild type ligand sequence.
4. A ligand according to claim 3 in which the said modification comprises the substitution of one or more amino
20 acids in the wild type ligand sequence.
5. A ligand according to claim 4 in which the said modification comprises the substitution of a single amino acid in the wild type ligand sequence.
25
6. A ligand according to claim 4 or 5 in which the said modification comprises the substitution of one or more amino acids in the wild type ligand sequence with alanine.
- 30 7. A ligand according to claim 4 or 5 in which the said substitution is to valine.
8. A ligand according to any preceding claim in which the wild type ligand is myosin light chain kinase.
35
9. A ligand according to claim 8 wherein the said modification is a single or multiple amino acid substitution at positions 9, 14, or 20 in the sequence of myosin light

chain kinase.

10. A ligand according to anyone of claims 1 to 7 in which the wild type ligand is mastaporan.

5

11. A molecule comprising i) a ligand as described in any one of the preceding claims, linked chemically to ii) another moiety.

10 12. A molecule according to claim 11, when the said moiety is a substrate for a chemical reaction.

13. A molecule according to claim 11, when the said moiety is a label, toxin, antibody or other targeting molecule.

15

14. A method of enhancing the binding affinity of a ligand capable of binding a calcium dependent binding protein, comprising the step of modifying the amino acid sequence of the wild type calcium dependent binding protein.

20

15. A binding pair comprising a calcium dependent binding protein and a ligand according to any one of claims 1-13.

16. Use of a ligand according to any one of claims 1-13 in conjunction with a calcium dependent binding protein, as a binding pair.

25

17. A ligand according to any one of claims 1-13 for use in therapy.

30

18. Use of a ligand according to any one of claims 1-13 in the manufacture of a pharmaceutical composition for the imaging, diagnosis or treatment of tumours.

FIG. 1

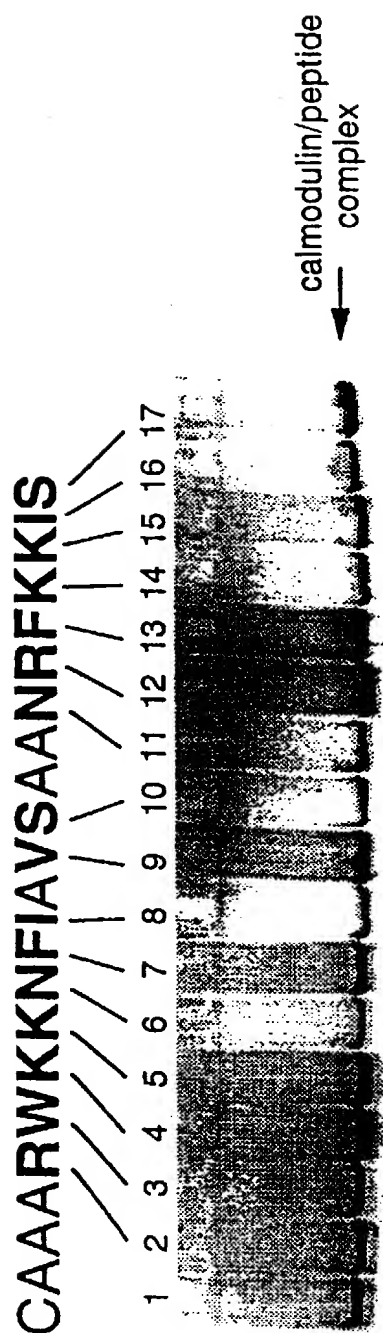


FIG. 2

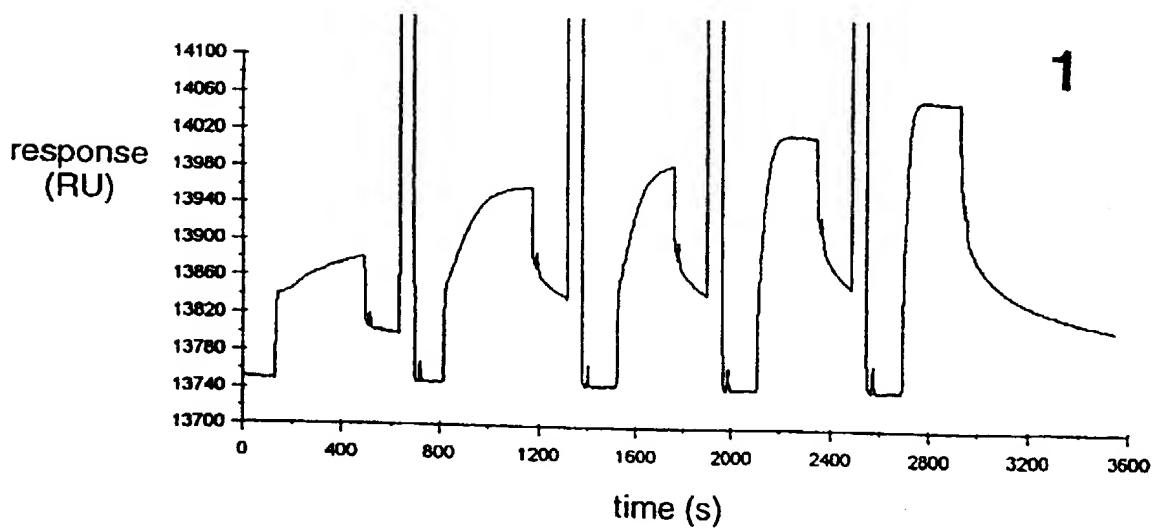
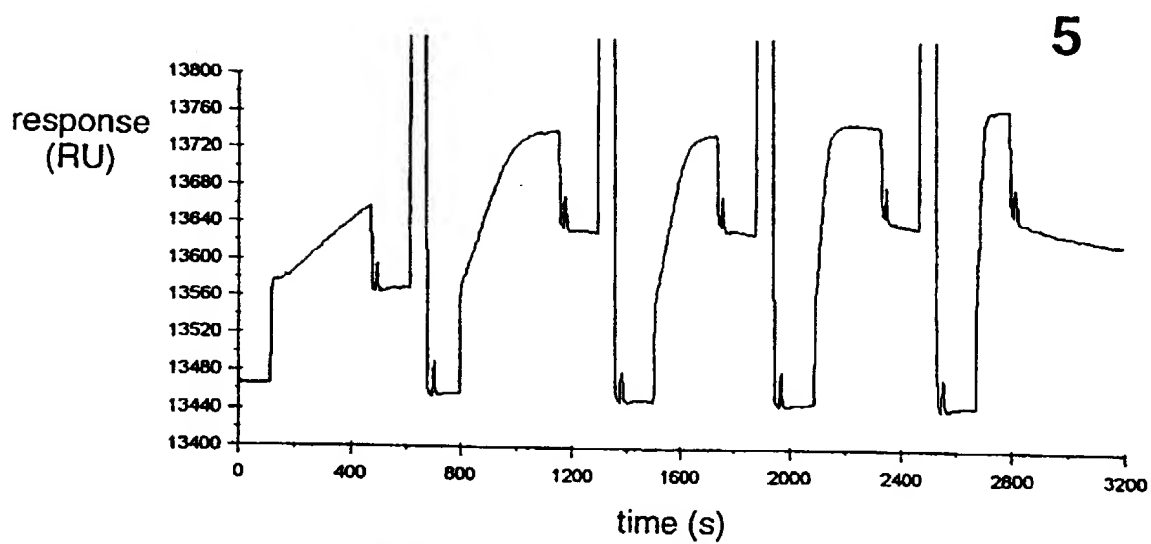
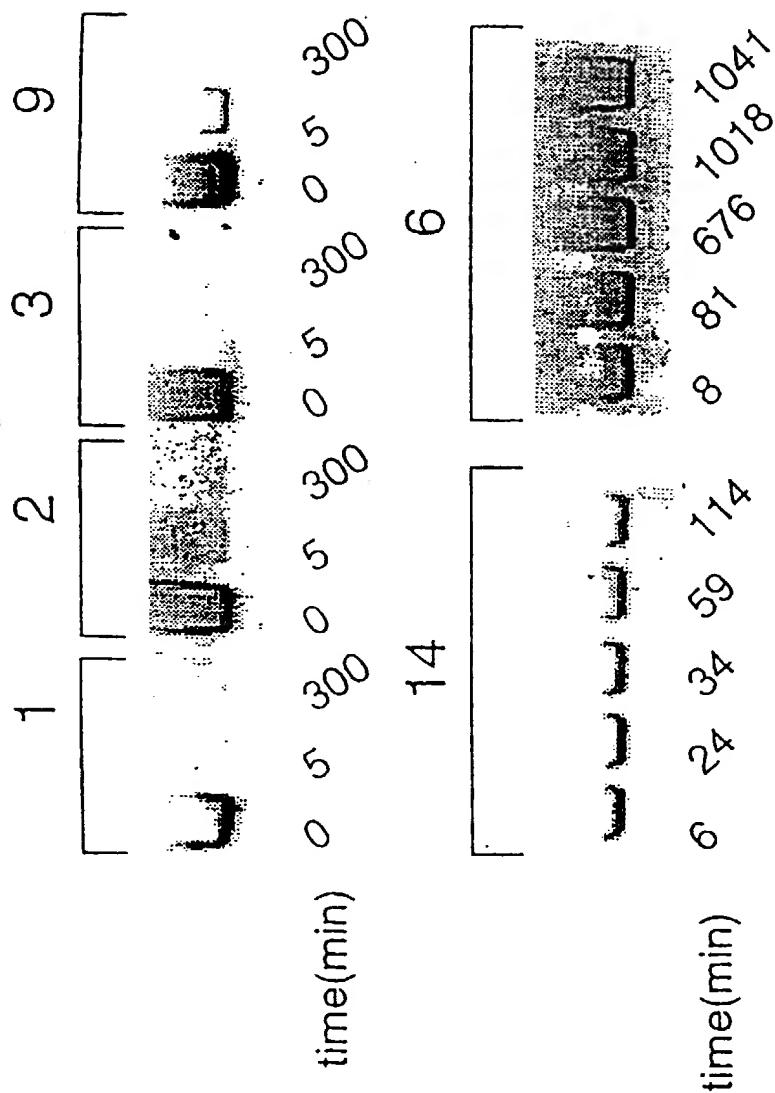


FIG. 3



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01152

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/12 C07K14/435 A61K38/16 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. MOL. BIOL., vol. 258, no. 1, 1996, NEW YORK, pages 6-13, XP002037716 S MONTIGIANI ET AL.: "Alanine substitutions in calmodulin-binding peptides result in unexpected affinity enhancement" see the whole document --- -/--	1-18

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

13 August 1997

Date of mailing of the international search report

26.08.97

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INTERNATIONAL SEARCH REPORT

I. International application No.

PCT/GB 97/01152

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-18
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Due to the extremely obscure wording of the claims, which i.a. characterise the claimed products by a result to be achieved, in contrast to Art. 6 PCT, the search was limited to the subject matter of claim 1 only insofar as further defined by claims 2,8 and 10 taken together
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01152

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BIOCHEMISTRY, vol. 33, no. 43, 1 November 1994, EASTON, PA US, pages 12807-12820, XP002037717 K TÖRÖK & D R TRENTAM: "Mechanism of 2-chloro-(epsilon-amino-Lys75)-[6-[4-(N,N- diethylamino)phenyl]-1,3,5-triazin-4-yl] calmodulin interactions with smooth muscle myosin light chain myosin light chain kinase and derived peptides" cited in the application see table 1</p>	1-18
X	<p>--- JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 28, 14 July 1995, MD US, pages 16848-16853, XP002037718 J K KRUEGER ET AL.: "Intrastereic regulation of myosin light chain kinase" see table 1</p> <p>-----</p>	1-18